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THE FINE STRUCTURE OF THE AMEBOCYTE IN THE BLOOD OF *LIMULUS POLYPHEMUS*. I. MORPHOLOGY OF THE NORMAL CELL

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ABSTRACT

The fine structure of the amebocyte (hemocyte) in the blood of *Limulus polyphemus* was reinvestigated regarding its normal state to resolve existing discrepancies in reporting before proceeding to studies of its participation in blood coagulation. The ovoid cell has all the organelles of a well equipped, metabolically active cell; having a nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum, and ribosomes. However, no mitotic figures were seen. The most distinguishing characteristic of the cell is the presence of a population of large granules (predominant) and small, asymmetric dense granules (not always present). The large granules originate from the Golgi apparatus and the matrix of the developing granules undergoes a wide range of transitional patterns before assuming the mature dense condition. The transitional stages are interpreted as formative of new granules rather than stages of degranulation as previously reported. The small, asymmetric dense granules are considered to be a type of granule distinct from the larger more predominant type of granule.

INTRODUCTION

The blood coagulation mechanism of *Limulus polyphemus,* the horseshoe crab, is notable because of its marked sensitivity to the endotoxins produced by gram-negative bacteria. The initial observations leading to this important discovery were reported by Frederik B. Bang (1956). Within two decades the original investigations have been extrapolated into many areas of basic and clinical research (Cohen, 1979). Clot formation is dependent on a characteristic hemocyte that discharges its granules as part of the phenomenon (Levin and Bang, 1964a, b; Levin, 1967; Murer *et al.,* 1975; Ornberg and Reese, 1981). In preparation for ongoing studies of factors that may either stimulate or inhibit clot formation, the fine structure of the normal hemocyte was examined in order to obtain control or base line observations. The most complete description to date of the fine structure of the native, unstimulated hemocyte in *Limulus* has been provided by Dumont *et al.* (1966). Our observations and, particularly, our interpretations differ sufficiently from theirs and warrant the new study of the normal cell reported here. Since the *Limulus* hemocyte is motile (Armstrong, 1980), it is customarily referred to as an "amebocyte." That nomenclature is retained here. For a comparative review of the blood cell types in a range of invertebrates, including insects, see Ratcliffe and Rowley (1979).

MATERIALS AND METHODS

Horseshoe crabs (*Limulus polyphemus*) were obtained from the Marine Resources Department of the Marine Biological Laboratory, Woods Hole, Massachusetts, and

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kept, with feeding, in running seawater a week or more before being used. Fixing of the normal amebocytes in the blood was performed as described below.

A short 14 gauge hypodermic needle was thrust through the cephalothorax-opisthosoma flexure into the cardiac space. The blood expressed by cardiac pressure was allowed to flow directly from the needle to form a thin layer in the bottom of a petridish filled with fixative. The flexure was first cleansed with alcohol. The needle and the dishes were cleansed and heat sterilized at 180°C for 4 hours to eliminate possible endotoxins. The resulting clot was loosened and the upper surface layer, which was fully exposed to fixation, was dissected free to a depth of about 1.5 mm and trimmed prior to postosmication.

The primary fixative that proved best was 2.5% glutaraldehyde in 0.065 *M* Millonig phosphate buffer to which 0.7% tannic acid was added and the pH adjusted to 7.4. The tannic acid was the low molecular weight product manufactured from a Turkish Aleppo nut gall in Belgium (Simionescu and Simionescu, 1976) and sold by Mallinckrodt., St. Louis, Missouri (stock number 1764 and specify lot EAH).

Fixation was initiated at room temperature for about one half hour and completed for an additional three to four hours in a refrigerator (4°C). The tissues were then rinsed several times in cold 0.065 *M* phosphate buffer and placed in a cold mixture (4°C) of 2% aqueous osmium tetroxide (1 part), 2% potassium ferrocyanide (1 part), and 0.13 *M* phosphate buffer (1 part). The osmication vials were transferred to a slanted, rotating table and, for 45 min, allowed to come to room temperature. A brief rinse in 0.065 *M* phosphate buffer was followed by repeated rinses in distilled water, then dehydration and embedment in Epon 812. Thin sections were stained with uranyl acetate and lead citrate.

Supplemental observations of living amebocytes were made utilizing video-enhanced contrast, differential interference contrast microscopy, as described previously (Richardson *et al.*, 1983).

Results

The *Limulus* amebocyte in its normal state is a prolate disc that is sometimes modified toward a fusiform or spindle shape. It is readily identified by having many large granules and a centrally located nucleus (Fig. 1). The cytoplasmic matrix itself is packed with small ribosomal-like particles. A prominent circumferential band of microtubules is present and probably serves as a physical aid to preservation of shape. The band is not illustrated here because it does not differ from the bands described in the blood cells of many other species (Meves, 1904; Fawcett, 1959; Fawcett and Witebsky, 1964; Dumont *et al.*, 1966; Nemhauser *et al.*, 1980).

The large membrane bound granules (Fig. 1) are the most prominent and characteristic feature of the amebocyte. In the context of electron microscopy, the granules might better be designated "bodies" because of their large size, but the classical histological term "granules" will be used here. Two types of membrane bound granules were observed. The major population is composed of large spherical, sometimes ovoid granules. A minor population is composed of smaller, ellipsoid granules. We refer to the former as "major granules" and the latter as "minor granules."

Most of the major granules have a dense, evenly stained matrix. However, a few exhibit varying degrees of density with the matrix ranging from unorganized clumps to swirls of rod-shaped material that are compacted and finally fused into the homogeneous matrix seen in the mature granule. These are most frequently found in the neighborhood of the Golgi complex and we interpret them as "transitional granules." *i.e.*, formative stages of the major granule (Figs. 2–5, 8).

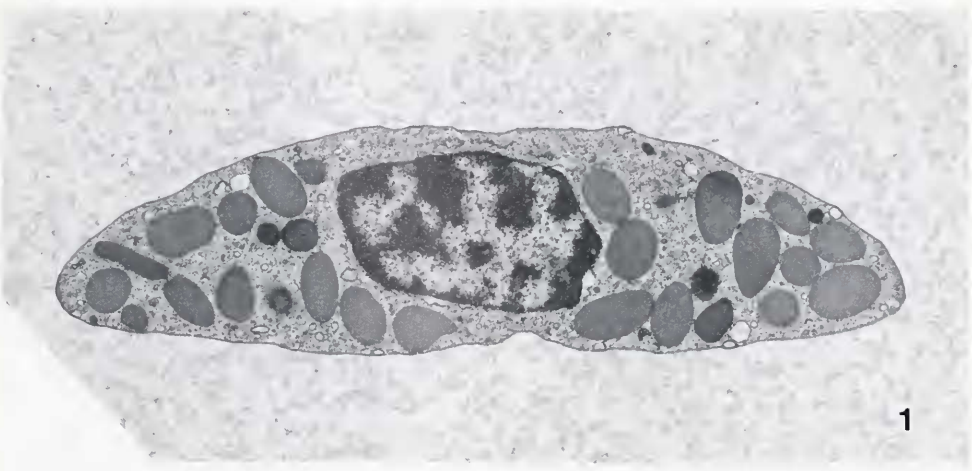

FIGURE 1. Longitudinal section of a *Limulus* blood amebocyte. In this section the cell is spindle shaped. A longitudinal section cut at right angles to this one would reveal a more oval shape. Note the characteristic large, homogeneous secretory granules. 7000×

The minor granules were not observed in all of the amebocytes but were seen often enough and are so unique in their morphology that they cannot be ignored (Figs. 6, 7). The central core of the minor granule is quite dense, more so than the matrix of the major granule. Most characteristic is a relatively clear zone at each end of the ovoid-shaped granule. No recognizable transitional stages of the minor granule were detected. There is no indication of its ultimate fate during blood coagulation. Very dense vesicles are sometimes produced by the Golgi apparatus and very probably contribute to formation of the minor granule.

Phase and differential interference contrast microscopic studies of living amebocytes revealed that the major granules have slow random movements. However, the minor granules often appear to move on long, linear paths which may be retraced immediately.

Many of the amebocytes have a well developed Golgi apparatus (Fig. 8). Due to the limitations of the plane of sectioning, it cannot be said that all of the amebocytes have an apparatus but indeed they are quite common. We never observed more than one Golgi apparatus in a cell though the cell may become quite large. Sometimes the complex may give rise to aberrant views dependent on plane of section. For example, a well developed apparatus may be curved into funnel shape (Fig. 9). Sections parallelling the top of the "funnel" demonstrate a circular or semi-circular stack of membranes (Fig. 10). Consequently, sections parallelling the long axis may present what appears to be two separate apparatuses associated at an angle. The *cis* face of the complex is to the outside of the funnel and the *trans* face is to the inside. The *cis* face is associated in normal fashion with elements of the rather sparse rough endoplasmic reticulum (Figs. 8, 9). The *trans* face, again in classic fashion, buds off small particulate filled vesicles that fuse into larger vesicles which finally form a transitional stage of the major granule type. Occasionally a few very dense vesicles are seen (precursors of the minor granule?).

Some of the secretory vesicles of the Golgi apparatus appear to leave the immediate zone and are found scattered throughout the cell. These may assemble in other parts of the cell to form transitional granules (Fig. 5). The evidence for this is suggestive but not conclusive. The transitional stages of the major granules that are sometimes

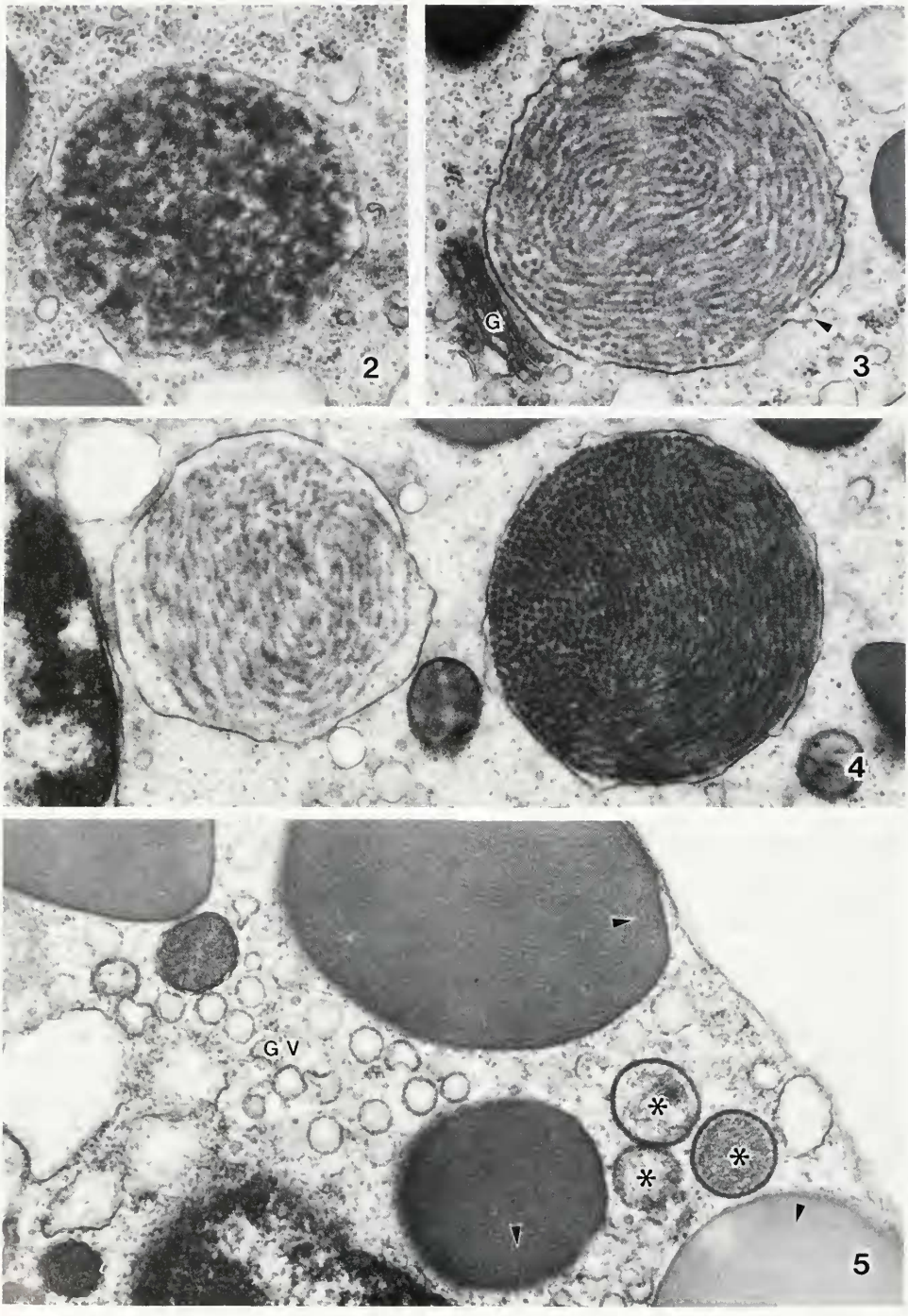

FIGURE 2. An early, amorphous stage in development of a secretory granule in the amebocyte of *Limulus*. 25,600×

FIGURE 3. An intermediate stage in the maturation of a secretory granule in the amebocyte of *Limulus*. The original flocculent material has condensed into roughly rod-shaped material. Note the attendant Golgi apparatus (G) and evidence of vesicle accretion (arrow). 22,490×

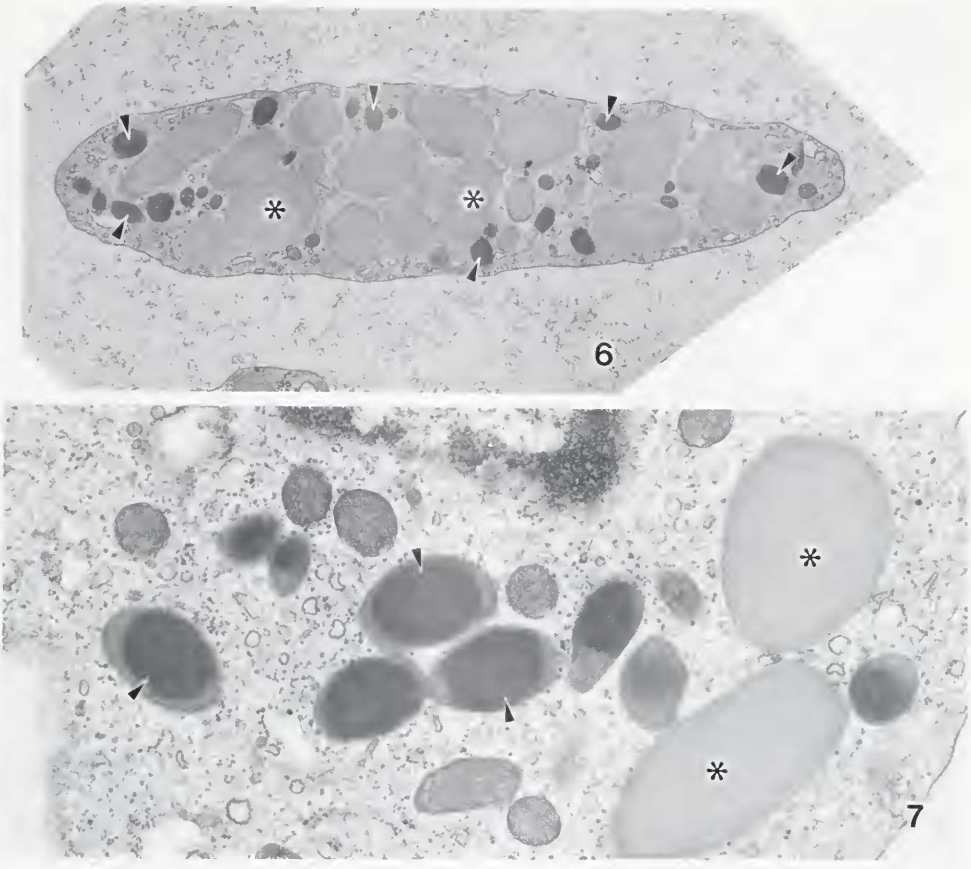

FIGURE 6. *Limulus* amebocyte showing both major (asterisks) and minor (arrows) granules. Note the marked density of the latter. 6140×

FIGURE 7. Detail of the two types of granules found in *Limulus* amebocytes. Major granules are marked with asterisks and the minor granules with arrows. 25,230×

distant from the Golgi zone might instead arrive there secondary to the random movement of the total population of granules.

Although not numerous, mitochondria are commonly found. Their dimensions vary from spherical (majority) to an elongated sausage-shape (minority). The matrix surrounding the cristae is quite dense and tends to obscure their membranes.

The amebocyte does not have a well organized endoplasmic reticulum. There is no identifiable tubular smooth endoplasmic reticulum. Irregular and sparse elements of the rough endoplasmic reticulum can be identified by the surface coating of ribosomes and the presence of more or less particulate material in the lumen. When seen,

FIGURE 4. Two intermediate stages in the maturation of the secretory granules in the amebocyte of *Limulus* blood. Left is an earlier stage and right is an advanced stage, the matrix being condensed into discrete rods. 25,790×

FIGURE 5. Granules of the amebocyte in *Limulus* shown in final stages of maturation. The matrix has become compacted into dense, intimately aligned rods. The rods have a less dense core (arrows). Typical Golgi secretory vesicles (GV) are present. Also seen are vesicles (asterisks) that may serve to accrete major secretory granules distant from the Golgi apparatus (see text discussion). They are characterized by a dense layer of material immediately under the limiting membrane. 34,220×

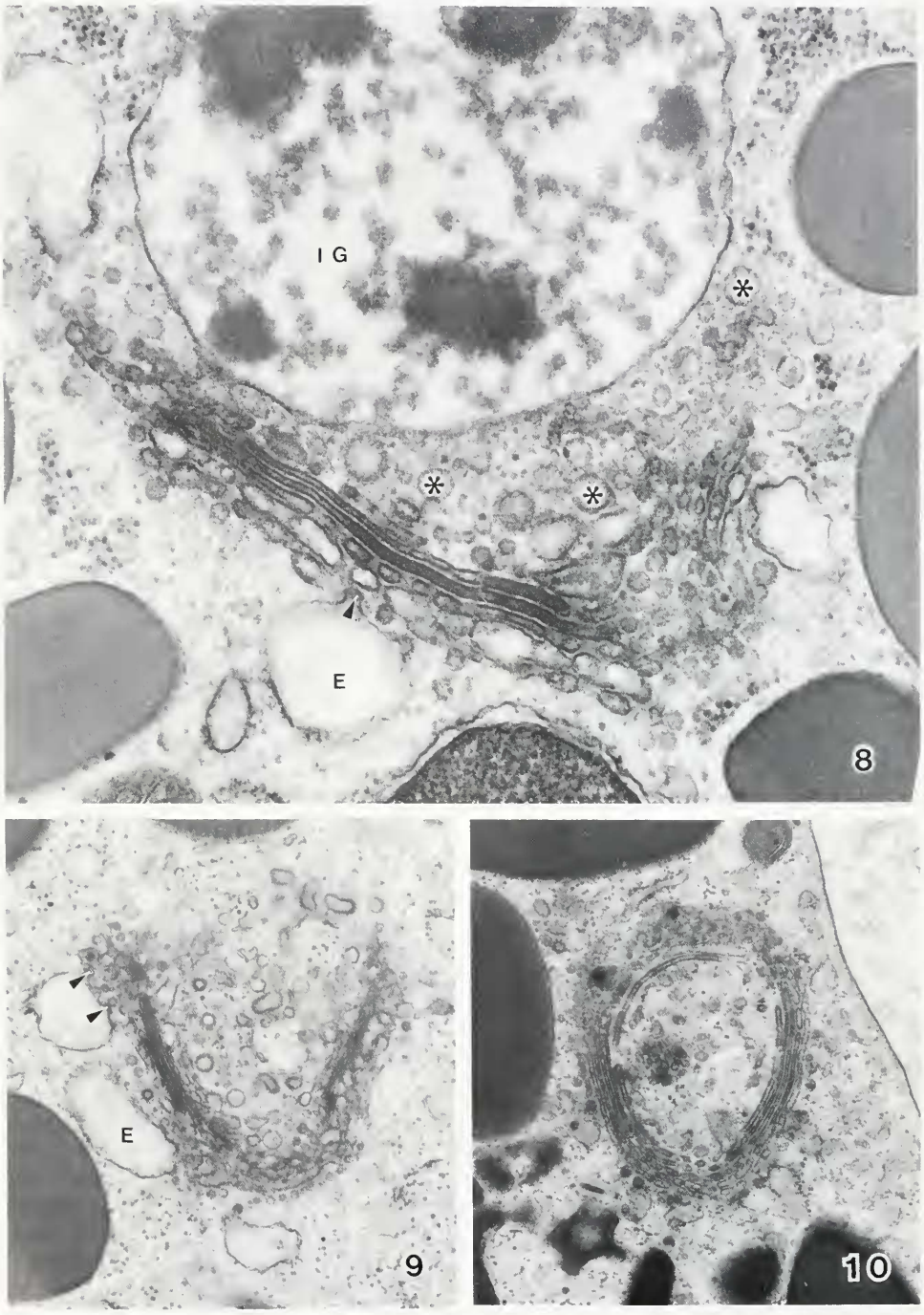

FIGURE 8. An active Golgi apparatus in the *Limulus* amebocyte. Rough endoplasmic reticulum (E) is budding material (arrow) into the *cis* face and secretory vesicles (asterisks) form on the *trans* face. A large immature intermediate granule (IG) is closely associated. 49,900×

the rough endoplasmic reticulum is usually adjacent to the cell surface, and, of course, in the Golgi zone. There is no obvious outer layer or zonation of the cell that might indicate preferential or obligatory location of rough endoplasmic reticulum. Its location near the cell surface may be merely the result of the random movement of the densely packed granules forcing it to that position.

The cytoplasmic space in the normal amebocyte is filled with densely packed particles. They frequently are joined in chains resembling the glycogen in the spinal glycogen body of chicks (Vye and Fischman, 1972). At higher magnification the particles resemble the alpha glycogen particles as classified by Drochmans (1962). The particles also morphologically resemble ribosomes (polysome type). Preliminary histochemical tests with a-amylase and RNAse controls indicate that the cytoplasmic particles are of a ribonuclear protein nature but that is still under investigation.

Discussion

The *Limulus* amebocyte is an interesting blood cell because it has all the organelles of a well equipped, metabolically active cell: a nucleus, mitochondria, Golgi apparatus, endoplasmic reticulum, and ribosomal particles. Nevertheless, it must be considered to be a "terminal" cell, origin unknown, because at no time were mitotic apparatuses seen in the circulating forms.

Dumont *et al.* (1966) present in good detail some of the morphology of the transitional granules described in this article. However, they concluded that the various stages represent chemical and morphological breakdown of the dense, homogeneous matrix into the dispersed, less dense vacuolar material that is finally expelled from the cell in the process of degranulation. Present evidence does not support that view and suggests that the transitional granules are formative stages of the dense major granule.

First, if the sequence of morphological stages represents a series of complex physicochemical reactions, it is unlikely that they could occur within the time frame of the almost explosive degranulation of the cell. Instead, the pleomorphic stages could represent an orderly, time consuming synthesis of material into the final major granule type. Secondly, the ubiquitous presence of all forms of the transitional granules adjacent to the Golgi zone suggests that they originate there. The occasional transitional granule in some other part of the cell could be explained by the random movement of all of the cellular granules. There is also some evidence that a few transitional granules may be assembled from small secretory vesicles that have migrated away from the Golgi zone.

As the result of experiments yet to be reported, it is our opinion that the major granule goes very abruptly from a dense phase to a finely particulate less dense phase as the cell degranulates. Normal time video-enhanced contrast, differential interference contrast cinematography by one of the authors (J.L.) has been made of endotoxin-free amebocytes. Under those specific conditions overall degranulation of the cell occurs during an extended period of time. However, the phase change undergone by any one granule is extremely rapid. In a fraction of a second a granule will expand

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FIGURE 9. A funnel shaped Golgi apparatus cut longitudinally giving the appearance of two apparatuses assembled in a "V." The rough endoplasmic reticulum (E) delivers material into the *cis* or receiving surface (arrows). 21,120×

FIGURE 10. A funnel shaped Golgi apparatus cut in cross section giving the appearance of a circular apparatus. See text for discussion. 23,050×

and lose its density. In some instances, degranulation is followed by a recoil type of movement of the cell.

That the phase change of the large granule from dense to finely particulate is always a rapid process, receives support from observations by Ornberg and Reese (1981) on ambeocytes that have been activated by a normal physiological agent, bacterial endotoxin. Using a slam-freeze cryofixation technique that immobilizes tissue in a few milliseconds (Heuser *et al.,* 1979), it was found that the phase change plus the event of exocytosis itself can occur in seconds with the phase change having no intermediate morphology(s) such as reported by Dumont *et al.* (1966).

Indirect support of our interpretation that the transitional granules are formative ones is provided by Bodammer (1978) in his study of the morphologically similar granular hemocytes of the blue crab, *Callinectes.* He interprets the various less dense granules as precursors of the mature dense granule. If it is correct that the transitional granules are indeed formative stages of the major granule and are normally present in the circulating blood, the descriptions in this report are more likely to represent the true undisturbed normal amebocyte.

The Golgi apparatus sometimes appears to be exceptionally large and active in that the layered membranes will fold around in the zone, assuming a circular or funnel shape. If the "funnel" is cross-sectioned near its mouth, the Golgi membranes appear to lie in a circle or semi-circle. If the "funnel" is sectioned longitudinally, there may be two Golgi layers of membranes tapering toward a common point. This perhaps accounts for the statement by Dumont *et al.* (1966) that several Golgi complexes may be seen in close association. We have no evidence of multiple apparatuses.

The nature and role of the oval shaped minor granules are unknown. Observations (by J.L.) of living cells reveal that an occasional small granule (minor granule?) may move in a more oriented fashion than the randomly moving larger granules. They can move in a straighter path and sometimes retrace the path. It could not be determined if such movement was associated with the circular marginal band of microtubules, there being no obvious association between the two components at the fine structural level. The clear areas of the oval-shaped granule might indicate a potential for oriented movement but we have no way of testing this possibility. Dumont *et al.* (1966) reported that small granules invade the pseudopods and retreat during active degranulation of the cell.

Many previous studies of *Limulus* have described the cytoplasmic granules (Levin and Bang 1964b; Muerer *et al.,* 1975; Ornberg and Reese 1981; Dumont *et al.,* 1966), but relatively little attention has been given to the possibility that different populations of granules are present within the cells. From the distinct differences in the morphology and the absence of detectable morphological transitions between the minor and major granules, we suggest that the *Limulus* amebocyte has two types of granules, one of which is not always present. By way of serendipidy, evidence was obtained that there may be a chemical difference between the two. The a-amylase enzyme used as a specific test for the possible glycogen nature of the cytoplasmic particles produced an unanticipated reaction in the minor granules that was not observed in the major granules. There is no currently available explanation for the particular reaction. Accordingly this subject is under further investigation.

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